ASSESSMENT OF HUMAN PAPILLOMA VIRUS-RELATED DISEASE

FIELD OF THE INVENTION

The present invention is generally related to the field of cytological and molecular assays and specifically to the area of assays for the assessment of disease using a sensitive assay for diagnosis and prognosis of HPV-induced carcinoma.

BACKGROUND OF THE INVENTION

The detection and diagnosis of disease is of obvious importance for the treatment of disease. Numerous characteristics of diseases have been identified and many are used for the diagnosis of disease. Many diseases are preceded by, and are characterized by, changes in the state of the affected cells. Changes can include the expression of viral genes in infected cells, changes in the expression patterns of genes in affected cells, and changes in cell morphology. The detection, diagnosis, and monitoring of diseases can be aided by the assessment of such cell states.

An aspect of the present invention relates to human papilloma virus (HPV), which induces benign epithelial proliferations of the skin and mucosa in humans and is associated with anogenital neoplasias and carcinomas. The intact DNA of HPV is supercoiled and thus resembles an endless loop of twisted telephone handset cord. Inside this shell, the viral DNA is packaged in and around proteins from the cell nucleus, histones, and associated peptides, into a structure that resembles cellular chromatin. (Turek, (1994)). Human papillomaviruses characterized to date are associated with lesions confined to the epithelial layers of skin, or oral, pharyngeal, respiratory, and, most importantly, anogenital mucosae. Specific human papillomavirus types, including HPV 6 and 11, frequently cause benign mucosal lesions, whereas other types, HPV 16, 18, and a host of other strains, are predominantly found in high-grade lesions and cancer. All human and

animal papillomaviruses appear to share a similar genetic organization, although there are differences in the functions of individual viral genes and in their regulation. The most common genital HPV type associated with cervical carcinoma, HPV 16, has been studied most extensively.

All large open reading frames (ORFs) in HPV are on one DNA strand. Papillomaviral mRNAs appear to be transcribed solely from a single strand in infected cells. The viral genome can be divided into three regions, the upstream regulatory region (URR), or long control region (LCR), containing control sequences for HPV replication and gene expression, the viral early gene region, encoding, among others, the E2, E6 and E7 genes, and the late region, encoding the L1 and L2 genes. (Turek, (1994)).

HPV gene expression in high-grade premalignant disease or cancer appears restricted to the early genes, possibly due to cellular differentiation arrest induced by the viral E6 and E7 genes. In comparison to active HPV infection, E6 and E7 gene control in cancer is deranged by mutations in the viral URR and, in integrated viral fragments, by the disruption of the viral E2 gene, stabilization of E6 and E7 mRNAs, and influences at the cellular integration site.

Because the E2 gene is disrupted or inactivated in integrated HPV fragments in invasive cervical carcinomas (Cullen, (1991); Dürst, (1985); Matsukura, (1989); Schneider-Gädicke, (1986); Schwarz, (1985); Wilczynski, (1988)), it has been predicted that loss of E2 bestows a selective growth advantage to the infected cell because of uncontrolled E6 and E7 expression (Schneider-Gädicke, (1986); Schwarz, (1985)). Indeed, cervical cells containing replicating HPV genomes rapidly segregate and are outgrown in culture by cells that contain integrated viral genomes (Jeon (1995)), but the underlying mechanism(s) have remained unclear until recently. The full-length HPV 16 E2 gene products are strong transcriptional activators comparable to HPV 1 E2 at some viral as well as at simple, synthetic promoters (Demeret (1994); Ushikai (1994)).

Genes E6 and E7 are considered to have oncogenic activity. The encoded proteins interact with and disturb the physiologic functions of cellular

proteins that are involved in cell cycle control. The E6/E7 proteins of HPV 16, 18 or related types are most efficient in this regard. Some of these activities lead to genetic instability of the persistently infected human cell. This enhances the probability of mutations in cellular proto-oncogenes and tumor suppressor genes and thus contributes to tumor progression. Mutations in cellular genes devoted to the intracellular surveillance of HPV infections, integration of viral DNA, and deletions or mutations of viral transcription control sequences lead to a significantly increased expression of the E6/E7 genes, which is a consistent characteristic of high-grade intraepithelial neoplasia and cancers. The genetic instability caused by viral oncoproteins and the autocatalytic increase in oncoprotein expression caused by mutations in the viral and cellular genome identify the virus as a major driving force of progression to carcinoma.

Individual types of human papillomaviruses (HPV) which infect mucosal surfaces have been implicated as the causative agents for carcinomas of the cervix, anus, penis, larynx and the buccal cavity, occasional periungal carcinomas, as well as benign anogenital warts. The identification of particular HPV types is used for identifying patients with premalignant lesions who are at risk of progression to malignancy. Although visible anogenital lesions are present in some persons infected with human papillomavirus, the majority of individuals with HPV genital tract infection do not have clinically apparent disease, but analysis of cytomorphological traits present in cervical smears can be used to detect HPV infection. Conventional viral detection assays, including serologic assays and growth in cell culture, are not commercially available and/or are not suitable for the diagnosis and tracking of HPV infection. Papanicolaou tests are a valuable screening tool, but they miss a large proportion of HPV-infected persons.

Thus, it is an object of the present invention to provide a method for assessing the stage of HPV-based disease.

It is another object of the present invention to provide an assay that can be combined with other assays to improve the accuracy and reliability of prognostic and diagnostic assessments of HPV-based disease.

It is a further object of the present invention to provide a method for assessing the risk that a patient infected with HPV will develop HPV-based disease.

It is another object of the present invention to provide a method for stratifying patients who are currently HPV-infected but without detectable HPV-based disease into those at risk for progression to disease and those not at risk for progression to disease.

It is also an object of the present invention to provide a method for identifying treatment regimes for patients having HPV-based disease.

Yet another object of the present invention to provide a method for monitoring the effectiveness of treatment of HPV-based disease.

A further object of the present invention to provide kits for assessing the stage of HPV-based disease.

Another object of the present invention to provide computer-based operation, analysis, and data management of assay data to assess the stage of HPV-based disease.

SUMMARY OF THE INVENTION

One embodiment of the present invention involves measuring the levels of expression of genes involved in a cell state, and comparing their expression to each other or to reference genes in a specific ratio, as an indication of the state of a disease in the cell sample. The present invention can be used to assess the stage or risk of a disease as indicated by the state of the cells. It can also be used to guide or assess the effectiveness of a therapy for a disease by identifying appropriate therapy based on the indicated cell state or by indicating any change in the state of cells subjected to the therapy.

In one form of the present invention, the stage and prognosis of a human papillomavirus (HPV) infection or HPV-based disease is assessed. This embodiment involves the measurement of the level of expression of two or more HPV genes. Genes for this purpose are the HPV E6, E7, L1, and E2 genes, although

other HPV genes such as E1, E4, E5, and L2 can also be used. It has been discovered that the level of expression of these genes, the ratio of expression of these genes to each other or to reference genes, or both, are indicative of the stage of HPV-based disease.

Gene expression levels are used according to this invention to assess the progression of HPV infection from benign to malignant growth. HPV infection progresses from CIN I through CIN III and finally to malignant cancer. These stages can be identified by the ratios of HPV genes. In particular, the transition from CIN I to CIN II/III, i.e., a transition to pre-malignancy, can be predicted when the ratio of the present invention exceeds one. A ratio of greater than 3 in the present invention indicates a transition from pre-malignancy to malignant cancer. Thus, a ratio below 1 indicates a low-level of CIN in an HPV infected cell. A ratio between one and about three indicates a high grade CIN (i.e., CIN III) or pre-malignant condition. And a ratio of over about three is an indication of an HPV induced malignancy.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph of detection of E6/E7 mRNA shown as the number of cells/well versus the number of E6/E7 mRNA/well.

Figure 2 is a graph of the sensitivity of an HPV L1 assay shown as the number of RNA molecules/well versus the signal-to-noise ratio minus 1.

Figure 3 is the probe sequence for HPV 16 L2 (SEQ ID NO: 2).

Figure 4 is the probe sequence for HPV 16 L1 (SEQ ID NO: 2).

Figure 5 is the probe sequence for HPV 16 E6/E7 (SEQ ID NO: 3).

Figure 6 is the probe sequence for HPV 16 E2 (SEQ ID NO: 4).

Figure 7 is the probe sequence for HPV 16 E4 (SEQ ID NO: 5).

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the identification and monitoring of

diseased cells. One method involves measuring the levels of expression of genes involved in a disease state, and comparing their expression to each other or to reference genes, as an indication of the state of the cells. Such measurements can be combined with other assays to increase the accuracy and reliability of the assessment of the disease state. One method of the present invention can be used to assess the stage of a disease as indicated by the state of the cells. This method can also be used to guide or assess the effectiveness of a therapy for a disease by identifying appropriate therapy based on the indicated disease state or by indicating any change in the state of cells subjected to the therapy.

Many diseases are characterized by specific cellular phenotypes and gene expression patterns. For example, neoplastic and cancerous cells generally exhibit certain distinctive morphologies and growth characteristics. Molecular characteristics, such as gene mutations and gene expression patterns are also a good indicator of disease progression. Virally infected cells can exhibit different morphologies and gene expression patterns, including expression of viral genes. Using the present invention, the characteristics of the cell state, such as changes in cell morphology or expression of genes can be determined from a patient sample.

The characteristics to be detected are generally specific to the cell state of interest and the disease suspected of being present in the cell sample. Such characteristics can be generally divided into two types, cytological characteristics and molecular characteristics. As used herein, cytological characteristics are characteristics such as, for example, overall cell shape and appearance. The primary identification and classification of many neoplastic and cancerous cells has traditionally been accomplished using cytological characteristics. Identification of cytological characteristics is generally slow, requires a relatively high level of training, and generally cannot be easily automated. As used herein, molecular characteristics are the presence and state of particular molecular species, such as proteins, nucleic acids, and metabolites. Such molecular characteristics are generally identified by detecting and measuring the particular molecules of interest.

The characteristics assayed can include additional or surrogate

marker characteristics that are not a direct cause or result of the disease but that are related to certain disease and cell states. Examples of such additional markers include polymorphic markers, human leukocyte antigens (HLA) such as B7 that predispose women for cervical carcinomas, oncogenes, p53 mutations, other cancer markers, oncosupressors, cytokines, growth factor receptors, and hormones. Such markers can be present in, or absent from, cells exhibiting state-or disease-specific characteristics, and such presence or absence can be indicative of, for example, a more severe or less severe disease state. These markers can be used in conjunction with the disclosed method to infer either higher or lower risk of neoplastic disease depending on the number of abnormal scores or the magnitude of change in quantitative markers.

Examples of disease states for assessment using the present invention include, but are not limited to neoplasias and cancer. Disease states of interest are HPV-based disease -- including HPV infection, cervical intraepithelial neoplasia (CIN), and cancer, atypical squamous cells of undetermined significance (ASCUS), warts, condylomata, epidermo dysplasia verruciformis and other skin diseases, laryngeal papilloma, oral papilloma and conjunctival papilloma.

An embodiment of the present invention is detection and measurement of the expression levels of certain HPV genes. An impressive amount of data has been accumulated over the past decade, showing that carcinoma of the cervix is associated with infection of certain types of HPV. Though the presence of HPV DNA in a precancerous lesion is indicative of an increased relative risk for cervical dysplasia and invasive carcinoma, it is still difficult to predict the clinical behavior of precancerous cervical lesions. Tumors arise due to the accumulation of genetic alterations which can activate oncogenes and/or inactivate tumor suppressor genes and/or genes involved in DNA damage recognition and repair.

The expression levels of E6 and E7 oncoproteins encoded by high-risk HPV types are a more sensitive and accurate measure of potential risk of an HPV infection developing into a cancerous lesion. The present invention measures the relative amounts of E6 and/or E7 expression levels and E2 and/or L1

in an HPV-infected lesion to determine the ratio of E6 and/or E7 to L1 and/or E2, where in this ratio is a direct measure of risk, and susceptibility to the development of a cancerous lesion. HPV expression can be measured by mRNA or protein levels in the cell.

In one aspect of the invention, the stage and prognosis of a human papillomavirus (HPV) infection or HPV-based disease is assessed. This embodiment of the present invention involves the measurement of the level of expression of one or more HPV genes discovered to be related to the stage and nature of HPV-based disease. Genes useful for this purpose include the HPV E6, E7, L1, and E2 genes. It has been discovered that the level of expression of these genes, the ratio of expression of these genes to each other or to one or more other genes, or both, are indicative of the stage of HPV-based disease. The level of expression is relative to other HPV genes, or the level of expression relative to a non-HPV gene, referred to herein as a reference gene. Such reference genes can be any appropriate gene (not encoded by HPV), and are, for example, housekeeping genes or other constitutively expressed genes. Examples of reference genes include actin genes, cytoskeletal genes, histone genes, tubulin genes, epidermal growth factor receptor genes, the normal p53 gene, the normal pRB gene, cyclin genes, ß-globin genes, and glucose-6-phosphate dehydrogenase genes. Expression of reference genes can be measured in the same cell as the level of HPV genes are measured or in neighboring cells in the same cell sample. In such a case, the reference gene is an internal control for gene expression.

The relationship of the relative level of expression of these genes to the state of cells infected with HPV is generally illustrated in Table 1 below.

Table 1

Medical Character	HPV-Based disease state	E6, E7, E6 + E7	E2	L1
benign	HPV-infected normal tissue	low	low to high	low to undetectable
benign	Low grade CIN i.e., CIN I	low to medium	low to high	medium to high
neoplastic	High grade CIN i.e., CIN II/III	medium to high	low to undetectable	low to undetectable
neoplastic	Cancer	medium to high	low to undetectable	low to undetectable

The HPV-based high grade CIN referenced in the table is the premalignant stage leading to cancer and low grade CIN is a productive viral infection that has little malignant potential but is a public health concern with respect to the spread of HPV infection. Normal tissue refers to cytologically normal tissue that is infected with HPV. Although not limited to this standard, one standard for establishing this lower limit of expression is a level below that detectable using the hybrid capture assay described in WO 93/10263 by Digene. As used herein, E6/E7 refers to E6, E7, or E6 + E7. The addition of genes, as with "E6 + E7," for example, refers to the combined expression of the added genes.

As can be readily discerned, each major disease state is represented by a unique expression pattern of these three sets of genes. Both of these conditions are regarded as serious medical aliments. Other relationships involving the relative level expression of other HPV genes (such as E1, E4, E5, and L2), and other, non-HPV genes, can also be used to assess cell state. For example, L2 and E4 are frequently associated with benign viral production diseases, and E1 is similar in profile to E2 and is often deleted in malignancies. Other relationships of expression for these HPV proteins can exist for other HPV-based diseases, and the disclosed method can be used to assess the state of such other diseases using the appropriate levels and ratios for that disease.

Using information about the levels and ratios of HPV genes in different cell states, the stage of the disease can be assessed in several ways. In

some cases, the presence or absence of detectable expression is indicative of the disease state in the infected cells. For example, a lack of E2 expression (when HPV is present) is indicative of high grade CIN or cancer. In other cases, a change or difference in expression of an HPV gene product can be indicative of change occurring in the infected cell state. For example, an increased level of expression of E6 and E7—relative to, for example, an earlier sample or a reference sample—may be indicative of high grade CIN or cancer. A change in the ratio of E6/E7 expression to E2 expression is used to identify low grade CIN or a shift from normal cells to low grade CIN. Many other combinations of comparisons are also possible, and other combinations can be derived from the information in Table 1 for use in the method of the present invention.

One way in which ratios of HPV genes can be related to HPV-based disease states is by reference to groups of HPV genes. For this purpose, group 1 genes or gene sets include E6, E7, and E6 + E7. Group 2 genes or gene sets include L1, L2, E4, and any combinations. Group 3 genes or gene sets include E1, E2, E5, and any combinations. Useful ratios of expression include ratios of members of group 1 to members of group 2 or group 3. Examples of theses ratios are (E6 + E7)/(L1 + L2), E6/L1, E7/L1, E6/L2, E7/L2, (E6 + E7)/L1, (E6 + E7)/L2), E6/(L1 + L2), and E7/(L1 + L2). For such ratios, a value of less than two is indicative of benign human papillomavirus infection or low grade intra-epithelial neoplasia. This type of infection is also classified as CIN I. HPV infections which progress beyond CIN I indicate cell transformation has occurred and cancerous growth has begun. These later infections (CIN II/III) have ratios of greater than 2. A ratio of expression of more than two is indicative of high grade intra-epithelial neoplasia or pre-malignant cancer. A ratio of expression of much more than two (i.e., exceeding 4 and up to infinity) is indicative of cancer. Preferred ratios for use in this invention are (E6 + E7)/L1, (E6 + E7)/(L1 + L2), (E6+E7+E2+E4)/(L1 + L2), (E6 + E7)/(E2 + E4), (E6 + E7)/E2, (E6 + E7 + E2-E4)/(L1 + L2).

There are several ways in which measured levels of expression of HPV genes can be compared and categorized. For example, where the presence or

absence of expression is indicative of the cell state. expression of the HPV gene is analyzed without reference to the expression level of other genes. Where the relative level of expression of an HPV gene is indicative of the cell state, the measured level of expression is compared, for example, to the level of expression of the same type of HPV gene in a different cell sample (such as an earlier cell sample from the same source or reference cells harboring HPV), to the level of expression of a different type of HPV gene in the same or a different cell sample, to the level of expression of a non-HPV reference gene in the same cell sample, or to the level of expression of a non-HPV reference gene in reference cells.

In one embodiment of the present invention, levels and ratios of expression of HPV genes are compared to the levels of the same genes in a cell line that contains HPV (such as HeLa or CaSki). Such cell lines provide a standard against which levels of expression of HPV genes in cell samples are compared. Such comparisons are used to assess and compare the absolute levels of expression of these HPV proteins with those in a standard or comparative cell line. Other cell lines useful for this purpose are non-cancerous cell lines infected with HPV 16 (such as W12) or HPV 31 (such as CIN-612; Meyers (1992)).

The levels and ratios of expression of HPV genes are also compared to reference genes, such as housekeeping genes or other constitutively expressed genes, in the same cells or in reference cells, such as a cell line. For example, the level of expression of the HPV gene and the reference gene is measured in the same cell sample. Such measurements provide an internal control of the overall expression level in a cell sample and are used to calculate a corrected level of expression for the HPV gene to allow more accurate comparisons of the level of expression between different cell samples. One form of correction is referred to as normalization. Thus, the level of expression of one or more HPV genes can be measured in two or more cell samples along with the level of expression of the same reference gene in each of the cell samples. The level of expression of the HPV genes is then normalized to each other based on differences, if any, between the measured level of expression of the reference gene in each of the samples.

In some stages of HPV-based disease, the expression of a particular HPV gene is low or undetectable. Such lack of detectable expression is used herein to identify patterns of expression that serve as prognostic or diagnostic indicators. The expression of other HPV genes is assessed in parallel or in the same assay to provide a control for the lack of expression of one or more of the genes to be assessed. This is accomplished by measuring the expression of the several HPV genes together or in parallel. For example, measuring the level of expression of the HPV E6, E7, L1, E4, and E2 genes in parallel is useful since at least one of these genes is expressed in all of the stages of HPV-based disease. In a preferred embodiment of the present invention, a ratio of E6/E7 to L1/E2 provides an indicator of likelihood of developing cancer from the HPV infection. In this way, indicative information is collected as well as providing an internal control. The presence of L1, L2, and E4 in combination is also indicative of benign disease while the absence of E1 and/or E2 is indicative of neoplastic potential.

The types of comparison described above can also be used with other genes and other disease states. That is, the measured level of expression of a gene of interest can be compared, for example, to the level of expression of the same type of gene in a different cell sample (such as an earlier cell sample from the same source or appropriate reference cells), to the level of expression of a different type of gene in the same or a different cell sample, to the level of expression of a reference gene in the same cell sample, or to the level of expression of a reference gene in reference cells.

Expression of genes of interest, such as the HPV E6, E7, L1, E4, and E2 genes, can be assessed using any suitable method. For example, RNA can be detected using hybridization, amplification, or sequencing techniques, and protein can be detected using specific antibodies. Many techniques for the specific detection of gene expression, by detection of expression products, are known and can be used with the disclosed method. One technique for detecting and measuring the level of expression of genes of interest is detection of RNA transcribed from the genes of interest. For the most reliable comparisons, expression levels that are to be

compared should be measured using the same technique and be performed in the same manner.

For hybridization detection of HPV nucleic acids, a mixture of probes specific for these sequences from different HPV types can be used. This ensures that the method will detect expression regardless of the type of HPV involved. For some purposes, it may be desirable to use probes designed for the sequence of a certain HPV type, or a mixture of probes for only some HPV types. Such probes may or may not be type-specific depending on the differences between the sequences of the HPV nucleic acids to be detected. One useful mixture for this purpose would include probes for HPV types more closely associated with a progression to cancer. The HPV types most commonly associated with cervical cancer are types 16 and 18.

Useful techniques for measuring the level of expression of a gene of interest in a cell sample include the hybrid capture technique described in WO 93/10263 by Digene, PCR *in situ* hybridization techniques described by (Nuovo, 1997)), branched DNA assays (Chernoff (1997)), transcription-mediated amplification (TMA); Stoflet (1988)), and polymerase chain reaction (PCR), ligase chain reaction (LCR), self-sustained sequence replication (3SR), nucleic acid sequence based amplification (NASBA), strand displacement amplification (SDA), and amplification with Qß replicase (Birkenmeyer and Mushahwar, (1991); Landegren, (1993)).

Numerous assays for the detection and measurement of gene expression products are known and can be adapted for the determination of the level of expression of genes of interest in the disclosed assay. For example, many of the techniques for the detection of HPV in general or expression of other HPV genes described below can also be adapted for use in the disclosed assay for the detection of expression of HPV genes E6, E7, L1, E4,and E2.

Many HPV detection and typing assays, which can be used in the disclosed method, are known, including assays involving Southern blots, dot blots, *in situ* hybridization, polymerase chain reaction, and solution hybridization. Mant

(1997) describes PCR assays used to identify DNA from specific HPV types. Cope (1997) describes a PCR-based test using a consensus primer and a Hybrid Capture assay (HCA) of detection of HPV types. The hybrid capture assay is also described in WO 93/10263 by Digene. The hybrid capture assay is a useful method for detection of HPV and for determining HPV type in combination with the disclosed assay.

Swan (1997), discloses an HPV detection assay exploiting the 5' to 3' exonucleolytic activity of Taq DNA polymerase to increase the signal from fluorescent dyes by releasing them from genotype-specific probes during PCR. Lizardi (1997), describes a method of detecting HPV using *in situ* hybridization with non-radioactive probes and visualization with conventional bright-field or fluorescence microscopy, or laser scanning confocal microscopy. Zehbe [1] (1997), describes a modified version of *in situ* hybridization for detection of HPV that involves signal amplification. Leiserowitz (1997), describes use of reverse transcriptase-polymerase chain reaction to detect HPV. Zehbe [2] (1997), describes a nonisotopic, enzyme-linked immunosorbent assay-based sandwich capture hybridization assay for HPV detection.

In one embodiment of the present invention RNA was analyzed directly by solution based procedures. The cells were first lysed by adding a proteolytic enzyme to the cells contained in wells of a microtiter plate. Non-limiting examples of enzymes for use in the present invention include proteinase K or Pronase. Cells can also be subjected to detergent lysis or osmotic lysis or a French Press. After incubation, biotinylated DNA probes were added to each well. The RNA:DNA hybrids were captured onto a solid phase by transferring to streptavidin coated microplates. Alkaline phosphatase-conjugated antibodies to RNA:DNA hybrids were added to each well in the hybridization microplate and signals were generated by adding a chemiluminescent reagent such as CDP-Star® with Emerald II (Tropix) to each well. The signal was read from the microplate. The solution based DNA analysis was performed similarly to the RNA analysis except that the microtiter plates were coated with anti-RNA:DNA hybrid antibodies

and the probes were RNA probes.

Other methods for detection and assessment of HPV infections that can be used in the disclosed method are described in U.S. Patent Nos. 5,415,995, 4,777,239, 5,484,699, 4,983,728, 5,527,898, 5,364,758, 5,639,871, 5,501,947, 5,665,533, 4,748,109, 5,623,932, 5,665,571 and 5,648,459. These are just examples of HPV detection and typing assays that may be used in combination with the disclosed molecular assay. Many of the techniques described above can also be adapted for use in the disclosed assay for the detection of expression of HPV genes.

The disclosed assay and other assays can also be sequentially combined. That is, first one type of assay can be performed, and then, depending on the results, another assay can be performed. For example, an assay to detect HPV (or HPV type) can be performed first, then, if HPV is present, the disclosed molecular assay can be performed. As another example, the disclosed molecular assay can be performed first, and if the results of the assay were indicative of a high grade CIN or cancer, a cytological assay or biopsy can be performed. Such sequential combinations are particularly useful for limiting more extensive testing to patients and samples that are identified as high risk.

Useful sequential orders for the assays are (1) an HPV assay, followed by an assay for one or more other markers, followed by a cytological or histological assay; (2) a cytological or histological assay, followed by an HPV assay or an assay for one or more other markers, followed by an HPV assay or an assay for one or more other markers (whichever had not been performed first); (3) a cytological or histological assay, followed by a combined or simultaneous HPV and marker assay; (4) a combined or simultaneous HPV and marker assay, followed by a cytological or histological assay; and (5) detection of HPV, detection of HPV type, an HPV assay, and a cytological or histological assay. Each combination of assays is followed by an assessment, using the combined assay results, of the cell state, disease state, patient status, patient prognosis, or other assessment as described herein.

Where the results of initial assays are either equivocal or suggest a

more severe stage of disease, further assays are useful to clarify and confirm the initial results. For example, where a cell sample is a mosaic with some cells benignly infected with HPV and others exhibiting high grade neoplasia or cancer, an assay measuring expression of HPV genes may give equivocal results. By following up with a morphological assay, the presence of the high grade neoplastic or cancerous cells can be established. In this case, the benefit of the disclosed method is that only some of the cell samples assayed (those with either equivocal or severe results) need be tested further.

The disclosed method can also be combined with treatment regimes. For example, results in the disclosed assay or method indicative of high grade CIN or cancer suggest that antiviral therapy will be ineffective since these stages of disease are often accompanied by integration of HPV into the genome. On the other hand, assay or method results indicative of normal or low grade stages of disease suggest antiviral treatments since HPV is generally not integrated at these stages. Antiviral treatments include, for example, drugs or therapeutic vaccines. Where results of the disclosed method indicate a benign cell state, treatment can be avoided altogether. The ability to make such assessments reliably and accurately is a significant benefit of the disclosed assay.

The disclosed method can include the combination of a molecular assay as described above with any other assay for assessing a disease or state of cells in a cell sample. For example, a molecular assay measuring the level or ratio of expression of HPV genes can be combined with cytological assays, histological assays, determination of the HPV type present, determination of the level of HPV present, assays detecting other cellular markers such as oncoproteins or tumor suppressors, or combinations of these assays. Such assays are known and are used for the diagnosis of HPV infection or HPV-based disease and assessment of the stage of disease. Results from a molecular assay and one or more additional assays can be combined to increase the reliability of any assessment, prognosis, diagnosis, or monitoring of HPV-based disease. This possibility of combination is a particularly useful aspect of the disclosed method since the HPV molecular assays

described above provide information about HPV-based disease that is distinct from other assays. Where multiple assays point in the same prognostic or diagnostic direction, the reliability of the assessment is increased. Useful combinations include a cytological assay and the disclosed molecular assay, an assay for determining HPV type and the disclosed molecular assay, and a combination of a cytological assay, a typing assay, an assay for detecting cancer markers, and the disclosed molecular assay. Combined assays can be performed in any order and in any temporal relationship. For example, various assays can be performed in parallel or simultaneously. Such assays can be performed in any manner such as on the same apparatus by the same person, with different apparatus, or in the same or different locations.

Cytological assays for use in assessing the stage of HPV-based disease are known and can be used in the disclosed method. The well established Pap smear and Hematoxylin & Eosin stains (H&E) are preferred examples. The use and analysis of Pap smears and H&E stains are well-known in the art.

A cell sample as the term is used herein is primarily a collection of cells from a patient. One method of obtaining cells is through non-invasive means, which is defined herein as obtained without the puncturing of a patient. Examples of non-invasive means are, for example, cell samples obtained from urine or a nasal, epithelial, cervical or other cell surface scrape. Patient cells can also be obtained by other means including, for example, needle biopsy or tissue biopsy.

The cell sample can be preserved in a collection medium which allows for a combination of two or more assays of different characteristics related to a cell state of interest. As used herein, the assay or assays refer to detection or measurement of specific characteristics, the results of which may be combined with other such measurements of other characteristics to an overall assessment of a cell suspected of being infected with one or more diseases. These assays may include, for example, a combination of morphological analysis and quantitation of a particular RNA or DNA or protein whose levels provide a specific indication of the presence or progression of a disease. Alternatively, for example, the collection

medium can be used to combine an assay identifying the morphology of cells in a cell sample with one or more assays identifying the HPV type involved, and, for example, identifying whether the HPV type identified is a high risk or low risk HPV type for the development of HPV-induced cell transformation and cancer.

For example, sources of cell samples for assessing HPV-based disease include cervix, vagina, vulva, anus, penis, larynx, buccal cavity, lymph nodes, malignant deposits in any part of the human body, and epidermis; all of which are known sites of HPV infection and pathology.

Cell samples for use in the present invention can be collected and stored in liquid medium. Examples of useful cell collection media are STM (Digene), PreservCyt® (Cytyc), and CytoRich™ (Autocyte). These media (PreservCyt® and CytoRich™) were developed for the collection of cytological samples but can be adapted for use with molecular assays.

Cell samples for use in the method of the present invention can be fixed or processed in any manner consistent with the assays to be performed. For example, both cytological and molecular assays can be performed using cells fixed on a solid substrate such as, for example, a slide. The requirements of the assays to be performed will generally identify the sample processing to be used.

The present invention can be conveniently performed using kits that include one or more of the materials needed for the method, such as reagents and sample collection and handling materials. For example, kits can include cell collection medium, sample preserving reagents, reagents for specific detection of DNA and/or expression products (RNA or proteins) of one or more of the E2, E4, E5, E6, E7, L1 or L2 genes, and sample handling containers. Useful reagents for detection of expression of the HPV genes are nucleic acid probes for those genes. A kit may also contain control samples or reagents, or reagents and materials for performing other assays to be combined with the disclosed assay. In addition, the kits can contain reagents for the separation of RNA and/or DNA from other cellular components.

The present invention can be performed using devices adapted to the

method. Numerous devices for performing similar assays are known and in use and can be adapted for use with the disclosed assays and method. For example, devices are known for automating all or a part of sample assays and sample handling in assays.

All or part of the disclosed method can be controlled or managed using special purpose computer programs. The data collected from the disclosed method, and data from any other assay used in combination, can be compiled, analyzed, and output in various forms and for various purposes using special purpose computer programs. Such programs can be used with, or combined into, other patient or data management computer programs. The usefulness of such a program increases with the number of measurements or assessments to be combined, and the relative importance of each type of measurement to the overall assessment. Computer programs for use with the disclosed method can be used on general purpose computers, or can be incorporated into special purpose computers or computerized devices for controlling the disclosed method, handling and analyzing data from the disclosed method or both.

EXAMPLES

The examples herein are meant to exemplify the various aspects of carrying out the invention and are not intended to limit the invention in any way.

Example 1: General Methods for Nucleic Acid Analysis.

The assay for nucleic acids follows in general principle the method for detecting HIV RNA by the Digene Hybrid Capture HIV Test, described in WO 93/10263 by Digene. Briefly, following lysis, 50 µl of probe mix (containing DNA biotinylated probe) was added to each well. The plate was sealed and incubated at 65 °C for 2 hours for hybridization to occur. After hybridization, samples were transferred to a strepavidin-coated microplate, and 25 µL of anti-hybrid antibody was added to each well. The plate was agitated at 1100 RPM, for 1 hour, at room

temperature. Wells were washed 6X times with 65 °C wash buffer, followed by one wash using distilled water. 100 µl of a chemiluminescent substrate was added to each well and the plate was incubated at room temperature for 30 minutes. The plate was then read in the DML 2000 luminometer. The data was then expressed as signal-to-noise. Using a calibration curve, the chemiluminescent signal generated by each specimen was converted into mRNA copies per cell. The assay described above can be run on either whole lysed cells or nucleic acid separated from other cellular components.

Example 2: Quantitation of HPV

This example illustrates the measurement of HPV E6/E7 expression for use in the disclosed method. A method for detecting and quantitating HPV mRNA, including E6/E7 and mRNA has been developed. This example measures expression in CaSki cells, but the method is generally applicable to other cell lines and clinical specimens. CaSki cells contain an integrated high-risk HPV-16 genome (about 600 copies/cell). CaSki cells were maintained in subconfluence in RMPI 1640 media containing 10% FBS and 10 mM sodium pyruvate. For this example, CaSki cells were grown to confluence and were removed from the dishes by treatment with 0.1% trypsin-0.5 mM EDTA. Using trypan blue, viable cells were counted under microscopy. Cells were seeded, in 10 µl volumes, at final concentrations of 10, 10^2, 10^3, 10^4, and 10^5 cells/well in a polystyrene, tissue culture treated, 96 well plate. Each dilution was performed and seeded in triplicate.

The cells were lysed with Proteinase K (30 units) in a Tris-EDTA-buffered SDS). The plate solutions (20 mM Tris pH 7.4, 20 mM EDTA and 0.5' was sealed, agitated for 30 seconds at 1100 RPM and incubated at 37 °C for 30 minutes. The test for HPV mRNA follows in general principle the method for detecting HIV RNA by the Digene Hybrid Capture HIV Test, described in WO 93/10263 by Digene. Briefly, following lysis, 50 µl of probe mix (containing E6/E7 DNA biotinylated probe) was added to each well. The plate was sealed and incubated at 65 °C for 1.5 hours for hybridization to occur. After hybridization.

samples were transferred to a strepavidin-coated microplate, and 25 µl of anti-hybrid antibody was added to each well. The plate was agitated at 1100 RPM, for 1 hour, at room temperature. Wells were washed 6X times with 65 °C wash buffer, followed by one wash using distilled water. 100 µl of a chemiluminescent substrate was added to each well and the plate was incubated at room temperature for 30 minutes. The plate was then read in the DML 2000 luminometer. The data was then expressed as signal-to-noise. Using a calibration curve, the chemiluminescent signal generated by each specimen was converted into mRNA copies per cell. The data for the direct detection of HPV mRNA from CaSki cells is shown in Figure 1. This method is exemplified by the detection and quantitation of E6/E7 mRNA, but has also been applied to quantitate other mRNA molecules (for example, HPV L1 mRNA) from CaSki cells (Figure 2).

Example 3: Quantitation of HPV mRNA
Using Preservative Collection Medium

CaSki cell line was trypsinized by incubating with 0.25% Trypsin-EDTA for 5 minutes at 37 °C. Cells were pelleted from the suspension by centrifugation at 800 rpm for 3 minutes in Sorvall RT 6000 centrifuge. Cell pellet was resuspended in 500 µl of 1X PBS and counted under microscope Trypan Blue solution. Cells were diluted to 50 and 500 cells/µl in 1X PBS. 10 µl of each cell concentration, including zero point (10 µl of 1X PBS) were spiked in 3 ml of PreservCyt reagent. 100 µl of Sample Conversion Buffer were added into each tub to help visualize the cell pellet. All samples were mixed well and were spun down at 3800 rpm for 15 minutes in Sorvall RT 6000 centrifuge. Supernatants were discarded and tubes were drained by inversion on the Kimtowels for 2 to 5 minutes on the bench. All pellets were resuspended with 50 µl of the lysis reagent (50 units of Proteinase K) and mixture was transferred into the plate coated with streptavidin. Plate was covered with the plate sealer and was incubated at 37 °C for 30 minutes (heat block) with agitation every 15 minutes.

50 µl of each E6/E7 RNA calibration were loaded in designated wells

at 0, 10^3, 10^4, 10^5,10^6,10^7 and 10^8 molecules/well to construct calibration curve for mRNA in the specimen. 50 µl of the probe mix was added into each well. Plate was covered with the plate sealer and was agitated for 1 minute at 1100 rpm on the bench top shaker. Samples were incubated at 65 °C for 1.5 hours (hybridization reaction) in the heat block. Plate was transferred into the bench top shaker and was incubated for 1 hour with agitation at 1100 rpm at room temperature (capture reaction). 25 µl of Detection Reagent 1 was added into each well and plate was incubated without agitation for 1 hour at room temperature.

The contents of the plate were discarded and the plate was washed vigorously six times with Wash Buffer at 65 °C and one time with deionized water at room temperature. The plate was drained into Kimtowels and 100 µl of the Detection Reagent 2 was added into each well. Plate was incubated for 30 minutes at room temperature covered from the light. At the end of incubation time, plate was read on the Digene DML 2000 luminometer and the data were expressed as signal-to-noise.

Example 4: Comparison of the expression of E6/E7 RNA and L1 RNA in cell lines containing episomal and integrated HPV 16 DNA

Cell lines tested

The following cell lines were examined according to the procedures outlined above.

HaCaT: an immortalized human keratinocyte cell line (Boukamp (1988))

SiHa: a human cancer cell line (Friedl, (1970))

W12: a non-tumorigenic human cervical keratinocyte cell line (Stanley, (1989))

HPV Infection Status

HaCaT cells were infected with HPV 16 by the procedure of White et. al. (White (1998)) to produce an episomal (non-integrated, total sequence, not spliced) HPV infection. Approximately 1 copy of HPV16 was present for every 40 cells. These cells are considered a representative of early stage infection or CIN I (cervical intraepithelial neoplasia). W12 cells contain approximately 100 copies of episomal HPV16 DNA and represent pre-malignant, immortalized cells or CIN II or CIN III. SiHa cells contain 1-2 copies of HPV16 integrated into the genome. These cells are considered to represent cancer.

Procedure

The RNA analysis was done according to Example 1 or the following procedure. Single stranded, biotinylated, DNA probes containing the specific HPV16 gene sequences were prepared. For HaCaT and SiHa cell lines, cells were grown to confluency, cells were harvested, and the total RNA was purified using the RNeasy kit (Qiagen Inc., Santa Clarita, CA). For W12, whole cells were used for analysis. RNA calibrators containing the complete HPV genome were prepared by transcribing (+) sense RNA from a plasmid containing the complete HPV16 genome with T7 RNA polymerase. The RNA was then diluted to 10^3, 10^4, 10^5, 10^6, and 10^7 copies per 50 µl. Aliquots of cellular RNA were diluted to 50 µl and then 50 µl of Probe mix (containing the biotinylated, single-stranded DNA probe) was added and hybridized to the RNA specimens for 2 hours at 65 °C. The hybridization reactions were transferred to a streptavidin coated microplate and 25 µl of Detection Reagent 1 was added to each well. (Detection Reagent 1 contains the alkaline-phosphatase – anti-RNA:DNA monoclonal antibody conjugate.) During a 1 hour incubation with shaking, RNA:DNA hybrids were captured onto the streptavidin coated plate and were simultaneously reacted with the anti-hybrid antibody conjugate. After several wash steps, a chemiluminescent substrate (Tropix CDP-star with Emerald) was added to the wells, and the light output was measured in a microplate luminometer after 30 minutes incubation at room temperature.

- Quantitation

The quantitation of HPV mRNA was performed as follows. The results from the RNA calibrators were used to construct standard curves. The regression equation was calculated from the logarithm of the copies versus the logarithm of signal to noise minus one [(S/N)-1)]. The regression equations were then used to calculate the number of copies of mRNA in the cellular RNA samples.

Ratio Results

The ratios of HPV 16 E6, E7, E2, E4, L1 and L2 were calculated for each cell type. The results are shown in Table 2. These results demonstrate that in an episomal, early stage infection the ratio of (E6 + E7)/L1 is about 0.7, in the pre-malignant immortalized cell line the ratio is about 4 and in the cancerous cell line the ratio approaches infinity.

Table 2.

	HaCaT	W12	SiHa
HPV Status	Episomal	Episomal	Integrated
Cell Status	Early stage infection	Pre-malignant, immortalized	Malignant
(E6+E7)/L1	0.68	4.00	∞*
(E6+E7)/(L1+L2)	ND	3.47	59.9
(E7+E6+E2+E4)/(L1+L2)	ND	6.96	70.6
(E6+E7)/(E2+E4)	ND	1.00	5.60
(E6+E7)/E2	ND	4.40	12.00
(E6+E7+E2-E4)/(L1+L2)	ND	1.58	59.10

*L1 gene transcripts were undetectable in SiHa cells. Therefore, ratios of other gene transcripts to the L1 gene transcript are infinitely large.

Publications cited herein and the material for which they are cited are specifically incorporated by reference.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

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I claim:

1. A method of detecting HPV-induced cell transformation in a patient infected with HPV comprising the steps of:

   - measuring HPV-expressed mRNA levels from a sample collected from said patient, said mRNAs comprising one or more of E6 mRNA, E7 mRNA, E2 mRNA E1 mRNA, E4 mRNA, L2 mRNA and L1 mRNA;
   - determining expression level of E6 and/or E7 and expression level of E2 and/or L1 such that a ratio of E6 and/or E7 to L1 and/or E2 is determined, wherein a ratio of greater than 2 is indicative of HPV-induced cell transformation and risk of neoplasia.

2. A method of predicting the onset of HPV-induced cancer in a patient infected with HPV comprising the steps of:

   - measuring a group I and a group II and/or a group III HPV mRNA from a sample collected from said patient;
   - determining ratio of group I mRNA level to group II and/or group III mRNA level wherein a ratio of greater than 2 is indicative of neoplastic onset.

3. A method of monitoring stage of HPV-induced disease in a patient infected with HPV comprising the steps of:

   - measuring HPV-expressed mRNA levels from a sample collected from said patient;
   - determining expression level of E6 and/or E7 and expression level of E2 and/or L1; and
   - determining a ratio of E6 and/or E7 expression level to L1 and/or E2 expression level wherein a ratio of greater than 2 is indicative or neoplastic change.

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4. A method of detecting HPV-induced cancer in a patient infected with HPV comprising the steps of:

- measuring HPV-expressed mRNA levels from a sample collected from said patient, said mRNAs comprising one or more of E6 mRNA, E7 mRNA, E2 mRNA and L1 mRNA;
- determining expression level of E6 and/or E7 and expression level of E2 and/or L1 such that a ratio of E6 and/or E7 to L1 and/or E2 is determined, wherein a ratio of greater than 4 is indicative of HPV-induced cancer.

5. A method of predicting the risk or onset of HPV-induced cancer in a patient infected with HPV comprising the steps of:

- measuring a group I and a group II and/or a group III HPV mRNA from a sample collected from said patient;
- determining ratio of group I mRNA level to group II and/or group III mRNA level wherein a ratio of greater than 4 is indicative of HPV-induced cancer onset.

6. A kit comprising at least two probes capable of detecting HPV RNA, wherein at least one probe is selected from the group consisting of E2, E4, E6 and E7, and at least one probe is selected from the group consisting of L1, L2, E1, E2, E4, and E5.

7. The kit according to claim 6, wherein at least one probe is a DNA probe.

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